Influence of Co-matrix Proton Affinity on Oligonucleotide Ion Stability in Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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In this article we investigated the role organic base co-matrices play in reducing oligonucleotide fragmentation during analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). The organic base co-matrix plays an important role in influencing the gas-phase behavior of desorbed oligonucleotides. No correlation was found between the solution pH values and the molecular ion stability of two model oligonucleotides. Instead, a direct correlation between the co-matrix proton affinity and the oligonucleotide molecular ion stability is seen. A co-matrix whose proton affinity is close to or greater than the proton affinity of the nucleobases can serve as a "proton sink." We propose that upon laser desorption/ionization, the co-matrix competes with the nucleobases of the oligonucleotide for additional protons from the matrix. When a co-matrix such as triethylamine is added, the co-matrix, rather than the oligonucleotide nucleobases, is the preferred site of proton transfer from the matrix. Titration of standard oligonucleotide matrices with several co-matrices of differing proton affinity demonstrates that the co-matrix mole fraction is an important factor in oligonucleotide molecular ion stability. When the mole fraction of the co-matrix approaches that of the matrix, nearly complete elimination of oligonucleotide fragmentation is seen. Control experiments utilizing pyridine, a co-matrix whose proton affinity is less than that of thymine or the phosphodiester backbone, demonstrate that the co-matrix plays an active role in oligonucleotide stabilization. Information on matrix:co-matrix interactions with these analytes should facilitate improvements in MALDI-MS of oligonucleotides. (J Am Soc Mass Spectrom 1998, 9, 668–675) © 1998 American Society for Mass Spectrometry

Matrix-assisted laser desorption/ionization (MALDI) has proven to be a versatile method for the generation of intact molecular ions from oligonucleotides and nucleic acids [1–3]. Among the strengths of MALDI-MS analysis of oligonucleotides are its speed, simplicity, and ability to characterize complex mixtures. Recent improvements in MALDI-MS have extended the upper mass range, detection limits, and mass accuracy of oligonucleotide analysis [4–6].

However, there are still two substantial experimental barriers to the routine analysis of oligonucleotides by MALDI-MS: the cation adduction problem and molecular ion instability. The cation adduction problem arises from the polyanionic character of oligonucleotides. Upon transfer from the condensed phase to the gas phase, cations are sequestered by the oligonucleotide to reduce the Coulombic interactions from the net negative charge of −1 at each nucleotide residue. When the sequestered cations are nonvolatile, such as the alkali metals, this process results in a broadly defined peak whose width and complexity reflect the degree of adduction. Cation adducts not only suppresses ion abundance, but they also shift the molecular ion envelope to higher mass values reducing mass measurement accuracies.

Previous work addressing this problem has focused on sample preparation. One approach involved precipitating the oligonucleotide with ammonium acetate yielding the ammonium salt of the oligonucleotide [7, 8]. Ammonia is then volatile enough to be lost during the desorption/ionization process yielding an unadducted molecular ion. Precipitation, however, is limited to large oligonucleotides or nucleic acids. The more traditional approach utilized in MALDI-TOFMS involves the use of cation exchange resin beads [9, 10]. The resin beads work similarly to the precipitation method in that alkali metal cations are exchanged for the more volatile ammonium or hydronium ions. An

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1044-0305/98/$19.00
PII S1044-0305(98)00049-X

Received January 12, 1998
Revised April 1, 1998
Accepted April 1, 1998

alternative approach for reducing the cation adduction problem is the use of a co-matrix. Pieles et al. showed that the addition of diammonium hydrogen citrate to a trihydroxyacetophenone matrix was an effective combination for reducing cation adducts and improving ion production [11].

Other groups have investigated the use of co-matrices as a means of improving the quality of the mass spectral data obtained from oligonucleotides. Currie and Yates investigated the use of ammonium salts at pH 7 as co-matrices [12]. They found that only those co-matrix additives that co-crystallize with the oligonucleotide/matrix sample solution are effective at improving ion production and gas-phase stability. Chen and co-workers showed that ammonium salts may be important in the ionization process as well as reducing cation adduct formation [13]. Cheng and Chan also investigated ammonium halide salts and drew the conclusion that the anionic and cationic portions of the salt each play an important role in the desorption/ionization process [14].

Recently, we reported the use of organic base [15, 16] co-matrices as a versatile approach to reducing or eliminating adducts, especially from extremely "salty" samples [17]. An additional benefit of our organic base co-matrix approach was the reduced fragmentation seen during the analysis of larger oligonucleotides. Understanding and remedying the underlying causes of oligonucleotide fragmentation can lead to improvements in the MALDI-MS technique. Expanding on an earlier study of Nordhoff et al. [18], Smith and co-workers have demonstrated that protonation of the nucleobase by the matrix results in cleavage of the *N*-glycosidic bond leading to loss of the base and formation of a carbocation at the 1′ position of the sugar [19–21]. Subsequent cleavages can then occur along the phosphodiester backbone. Unwanted fragmentation decreases the molecular ion abundance and can lead to difficulties in identifying the oligonucleotide of interest.

To date, research aimed at remedying the oligonucleotide ion stability problem has revolved around modifications to the oligonucleotide structure [21–24]. Nucleobase modifications include the replacement of a ring nitrogen with carbon in adenine or guanine [22, 23]. These 7-deaza purines were found to be more stable upon laser desorption/ionization, an effect attributed to the loss of the most favored site of protonation on the purine base. Sugar modifications, such as 2′-fluoro or 2′-hydroxy substitutions have been shown to increase oligonucleotide molecular ion stability [21, 24]. In that case, the improvement in oligonucleotide stability arises from destabilizing the carbocation intermediate, thereby increasing the barrier to base loss [21].

An aim of our work has been to develop remedies to the oligonucleotide fragmentation problem that do not rely on modification of the oligonucleotide structure. Here, we report on studies whose goal is understanding the role of organic base co-matrices in reducing oligonucleotide fragmentation. The organic bases investigated include triethylamine, piperidine, imidazole, and pyridine which have a wide range of proton affinity values. We show that co-matrices of sufficiently high proton affinity serve as "proton sinks" during the desorption/ionization process and inhibit nucleobase protonation thereby reducing or eliminating oligonucleotide fragmentation in MALDI-MS. This approach does not rely on modifications to the oligonucleotide structure and is compatible with the common oligonucleotide matrices currently in use.

Experimental

Oligonucleotide Synthesis and Purification

Reagents for oligonucleotide synthesis were obtained from Perkin Elmer/Applied Biosystems (Foster City, CA). Trityl-on oligonucleotides were synthesized using standard phosphoramidite chemistry on 1 μmol columns using a Perkin Elmer/Applied Biosystems Model 394 DNA/RNA synthesizer. After synthesis, the deprotected oligonucleotides were purified using oligonucleotide purification cartridges (OPC) purchased from Perkin Elmer to isolate the desired sequence from failure sequences. Purification is accomplished by washing the oligonucleotide and retaining the terminal dimethoxytrityl (DMT) protecting group. The DMT-protected oligonucleotide is retained on the OPC in 10% aqueous ammonium hydroxide. The OPC was washed with ammonium hydroxide to remove any impurities and failure sequences. The DMT group was then removed using 3% trifluoroacetic acid (TFA) and the remaining oligonucleotide was eluted with 20% acetonitrile. The eluate was evaporated to dryness on a LabConco centrivap (Kansas City, MO) and then reconstituted in 100 μL of nanopure water prior to analysis. If the OPC purification did not isolate the desired reaction product, the sample was further purified using reversed-phase or anion-exchange high performance liquid chromatography. After purification, the oligonucleotides were evaporated to dryness and reconstituted in deionized water at a concentration of 100 pmol/μL prior to analysis.

Sample Preparation

6-Aza-2-thiothymine (ATT), diammonium hydrogen citrate, 3-hydroxypicolinic acid (3-HPA), picolinic acid (PA), and 2′,4′,6′-trihydroxyacetophenone (THAP) were purchased from Aldrich (Milwaukee, WI). The ATT matrix solution consisted of a 1:1 ratio of 70 mM ATT and 20 mM diammonium hydrogen citrate (pH 3.68). The 3-HPA matrix solution consisted of an 8:1:1 ratio of 0.36 M 3-HPA, 0.41 M PA, and 0.22 M diammonium hydrogen citrate (pH 2.80). The THAP matrix solution consisted of a 2:1 ratio of 0.5 M THAP and 0.1 M diammonium hydrogen citrate (pH 3.94).

Triethylamine was purchased from Mallinckrodt (Paris, KY). Imidazole and piperidine were purchased

from Sigma (St. Louis, MO). Pyridine was purchased from Aldrich (Milwaukee, WI). All imidazole solutions were prepared in deionized water. The organic bases were desalted using AG501-X8(d) resin beads (Bio-Rad, Hercules, CA). Approximately 5 g of resin beads were added per 100 mL of solution and allowed to stir for 1 h.

Mass Spectrometry

A PerSeptive Biosystems (Framingham, MA) Voyager linear MALDI-TOF instrument with a nitrogen laser was used for the analyses. The samples were prepared as either a 3:1 (v:v) matrix:analyte ratio or a 3:3:1 (v:v:v) matrix:co-matrix:analyte ratio. Each sample was spotted on the MALDI sample plate and allowed to air dry prior to instrument insertion. Calibration of the instrument was carried out using dpA_5 and dpT_{16}. Each mass spectrum was an average of 20 to 25 laser shots and the laser power density was held constant at near threshold power density throughout these investigations.

Results and Discussion

Previous work reported by our lab demonstrated improved MALDI spectra of oligonucleotides when organic base co-matrices were used [17]. During those studies, we noticed a reduction of metastable fragment ions from larger oligonucleotides although the role of the co-matrix in those results could not be determined. The addition of a co-matrix could affect oligonucleotide ion stability in one of three ways: (1) adjustment of the solution pH to values more favorable for laser desorption/ionization; (2) the co-matrix could promote gas-phase cooling of the analyte [12]; or (3) the co-matrix could reduce protonation of the analyte. We chose two oligonucleotide homopolymers to use as model analytes for the following experiments. $d[(Tp)_9T]$ was chosen because homopolymers of thymidine are known to be insensitive to base protonation-induced fragmentation in MALDI-MS, thus this oligonucleotide could be used as a control. $d[(Ap)_9A]$ was chosen as a representative model of an oligonucleotide that undergoes fragmentation during MALDI-MS analysis.

Effect of Solution pH on Molecular Ion Abundance and Stability

Although there are several studies concerning the influence of solution pH on oligonucleotide ion production in ESI-MS [25, 26], there are few reports on pH effects in MALDI-MS [10, 27]. Hillenkamp and co-workers did demonstrate that acidic hydrolysis of oligonucleotides is not a measurable occurrence during sample preparation steps [10]. The first series of experiments were performed in order to determine whether the improvement in molecular ion abundance seen during addition of the co-matrix in our previous study [17] was due to the solution-phase properties of the matrix:co-matrix mixture.

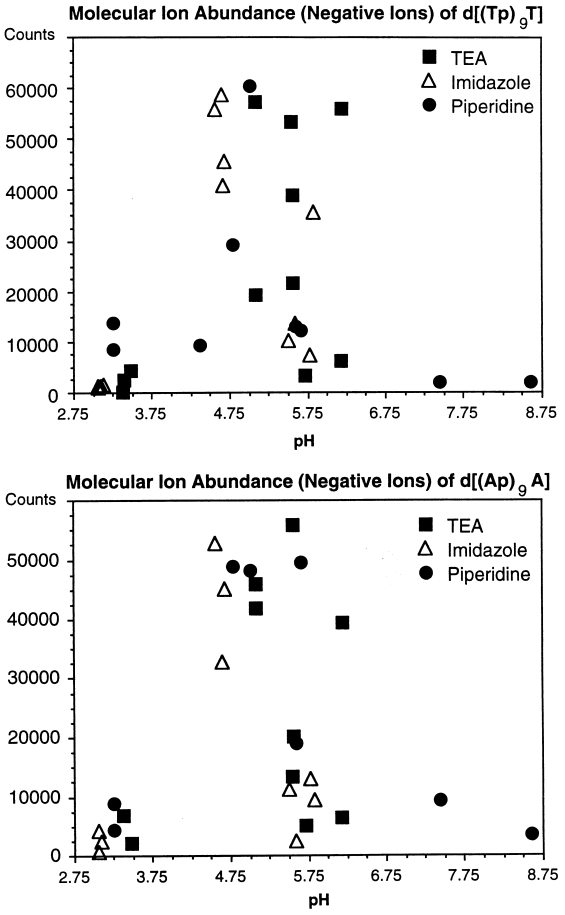

Figure 1. Negative ion mode MALDI-MS molecular ion abundances of (top) $d[(Tp)_9T]$ and (bottom) $d[(Ap)_9A]$ as a function of apparent solution pH for various matrix:co-matrix combinations. The data are plotted with respect to co-matrix identity.

The pH of each solution was determined prior to addition of oligonucleotide and sample spotting. The initial pH of each matrix was below 4 and adjustment to pH was because of the addition of different concentrations of the organic bases used. Because the solutions contain both deionized water and acetonitrile or ethanol, the pH readings obtained are only apparent pH values. No corrections to the pH reading for the presence of the organic component was made for the data reported here. No correlation was seen between co-matrix identity and molecular ion abundance for each of the matrix:co-matrix combinations studied. Figure 1 is a plot of the molecular ion abundance versus apparent solution pH for all of the matrix:co-matrix combinations investigated. Figure 1a is the molecular ion abundance for $d[(Tp)_9T]$ and Figure 1b is the molecular ion abundance for $d[(Ap)_9A]$. No definitive statements can be made regarding matrix:co-matrix choice or solution pH from these data. However, it is clear that for both analytes, the molecular ion abundance tended to be highest at apparent solution pH values between 4 and 6.

It is instructive to note the chemical species present

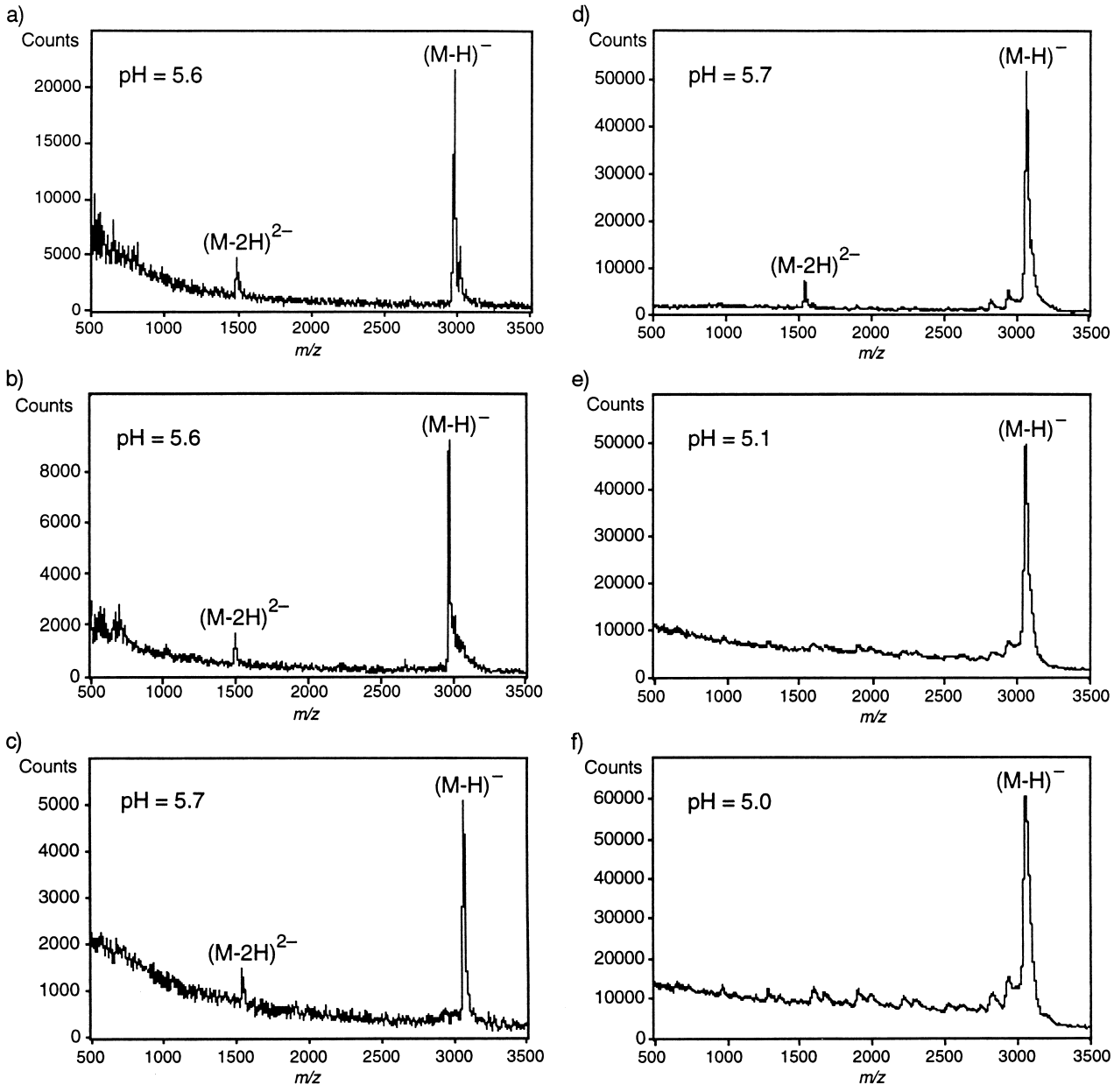

Figure 2. MALDI-MS mass spectra and apparent solution pH values of (**a**) d[$(Tp)_9$T] in THAP: piperidine solution (**b**) d[$(Tp)_9$T] in THAP:imidazole solution, (**c**) d[$(Ap)_9$A] in THAP:triethylamine solution, (**d**) d[$(Ap)_9$A] in THAP:piperidine solution, (**e**) d[$(Ap)_9$A] in ATT:triethylamine solution, and (**f**) d[$(Ap)_9$A] in ATT:piperidine solution.

in solution at pH values within the range of 3 to 8. The matrices and oligonucleotides are acidic with pK_a values near to or less than 4. The co-matrices are basic with pK_a values of 6.99 (imidazole), 10.7 (triethylamine), and 11.1 (piperidine). At the lower pH values (pH $<$ 4), the oligonucleotide and the matrix will exist in their neutral forms, thus oligonucleotide salts are less likely to be present in the crystal upon evaporation. At pH values between 4 and 7, the protonated co-matrix could be expected to form a salt with either the oligonucleotide or the matrix upon evaporation of the solvent. At higher pH values, only triethylamine and piperidine would be expected to form a salt with either the matrix or the oligonucleotide and as the pH increases further both of these co-matrices would not be protonated in solution and are probably lost during solvent evaporation. The results in Figure 1 are most likely due to these considerations.

The next series of investigations were aimed at determining the stability of the molecular ion signal as a function of solution pH. If solution pH values play a determining role in oligonucleotide fragmentation, then one would expect to find a correlation between the solution pH and molecular ion stability. Figure 2 is a

plot of several matrix:co-matrix combinations for d[$(Tp)_9$T] and d[$(Ap)_9$A] compared at similar solution pH values. Figure 2a, b is d[$(Tp)_9$T] in a THAP:piperidine matrix:co-matrix combination and a THAP:imidazole matrix:co-matrix combination, both at an apparent pH of 5.6. As one would expect, no fragmentation of the d[$(Tp)_9$T] molecular ion is seen. Figure 2c,d is d[$(Ap)_9$A] in a THAP:triethylamine matrix:co-matrix combination and a THAP:piperidine matrix:co-matrix combination, both at an apparent pH of 5.7. As seen in Figure 2c, no fragment ions are detected for the THAP:triethylamine combination. However, in Figure 2d several fragment ions are detected. Thus, the molecular ion *stability* and *abundance* appear to be independent of the solution pH. Differences in ion stability and abundance for particular matrix:co-matrix combinations are discussed further below. Figure 2e,f is a further demonstration of this fact. Figure 2e is d[$(Ap)_9$A] in an ATT:triethylamine matrix:co-matrix combination at an apparent pH of 5.1 and Figure 2f is d[$(Ap)_9$A] in an ATT:piperidine matrix:co-matrix combination at an apparent pH of 5.0. In each case significant fragmentation is evident, although the fragment ion abundances are much greater for the ATT:piperidine combination (Figure 2f) as compared to the ATT:triethylamine combination (Figure 2e). These results suggest that some other factor influences oligonucleotide molecular ion stability when organic base co-matrices are utilized.

Effect of Co-matrix Proton Affinity on Molecular Ion Stability

The next series of experiments were aimed at determining whether the gas-phase properties of the co-matrices were the important factors governing molecular ion stability. Hillenkamp and co-workers [18] and Smith and co-workers [19–21] have shown that metastable fragmentation of oligonucleotides in MALDI-TOFMS is due to nucleobase protonation. Base protonation leads to backbone strand scission and the reduction of molecular ion stability. It has previously been shown that protonation and strand scission does not occur in the MALDI matrix solution prior to the desorption/ionization step [10]. Thus, it is reasonable to assume that the proton affinities of the matrices, co-matrices, and nucleobases might play an important role in determining oligonucleotide stability.

The proton affinities of the nucleobases and deoxynucleosides are [28]: T 209.0, dT 224.9; C 225.9, dC 233.2; A 224.2, dA 233.6; G 227.4, dG 234.4 (all in kcal/mol). We have recently determined the proton affinities of the deoxynucleoside monophosphates and have found them to be approximately 1 kcal/mol lower than the corresponding deoxynucleoside proton affinities and we estimate the proton affinity of the phosphodiester backbone (i.e., the enthalpy change associated with protonating a neutral backbone) to be ~225.5 kcal/mol [29]. The proton affinities of the co-matrices used in this study are: imidazole 225.2 kcal/mol, piperidine 228.0 kcal/mol, and triethylamine 234.7 kcal/mol [30]. To date, only the proton affinity of 3-HPA has been determined [31, 32], although it is reasonable to assume that the proton affinities of each of the matrices used in this study are lower than the proton affinities of the nucleobases or deoxynucleosides (vide infra).

If the proton affinity is the determining factor in oligonucleotide stability, then the following predictions can be made on the basis of the known proton affinity values. Because the proton affinities of the matrices are lower than the proton affinities of the deoxynucleosides, oligonucleotide fragmentation should be evident to differing degrees in each matrix without any additional co-matrix additives. Furthermore, the addition of an organic co-matrix with a proton affinity higher than the proton affinities of the deoxynucleosides (e.g., triethylamine) should be more effective at reducing fragmentation than co-matrices having proton affinities less than or equal to the deoxynucleosides (e.g., imidazole).

d[$(Tp)_9$T] and d[$(Ap)_9$A] were analyzed using 3-HPA, THAP, and ATT matrices without any additional co-matrix (data not shown). d[$(Tp)_9$T] does not exhibit any fragmentation in any of these matrices. These results are to be expected as the proton affinity of the phosphodiester backbone is expected to be similar to or slightly higher than the proton affinity of the thymine base, thymine does not have a site that could easily accept a proton, and other researchers have reported similar results from studies of poly T [19]. However, d[$(Ap)_9$A] whose nucleobase has a higher proton affinity, does exhibit matrix-dependent fragmentation. Fragmentation is most prevalent in the ATT matrix. Base loss is seen in the THAP matrix and the 3-HPA matrix yields the cleanest mass spectral data.

On the basis of these results, the next experiments concentrated on the influence of co-matrix addition to oligonucleotides analyzed in THAP and ATT. Figure 3 is the mass spectral results from the addition of various amounts of co-matrix to THAP. In Figure 3a, 3 nmol of piperidine was added to 1.5 μmol of THAP. Significant base loss and some backbone cleavage products are observed in the mass spectrum. In Figure 3b, 3 nmol of triethylamine was added to 1.5 μmol of THAP. Although there is a loss of absolute molecular ion abundance (approximately a twofold decrease) similar to that found in ESI-MS experiments [15], the addition of a co-matrix with a higher proton affinity serves to significantly reduce the presence of fragment ions in the mass spectrum.

The proton affinity of triethylamine is nearly 7 kcal/mol greater than the proton affinity of piperidine and is on the order of the proton affinity of deoxyadenosine [233.6 kcal/mol (deoxyadenosine) versus 234.7 kcal/mol (triethylamine)]. If base protonation causes fragmentation of oligonucleotides in MALDI-MS, then the addition of a co-matrix of similar or higher proton affinity can serve as a "proton sink" and compete with the nucleobase for protons from the matrix. When the

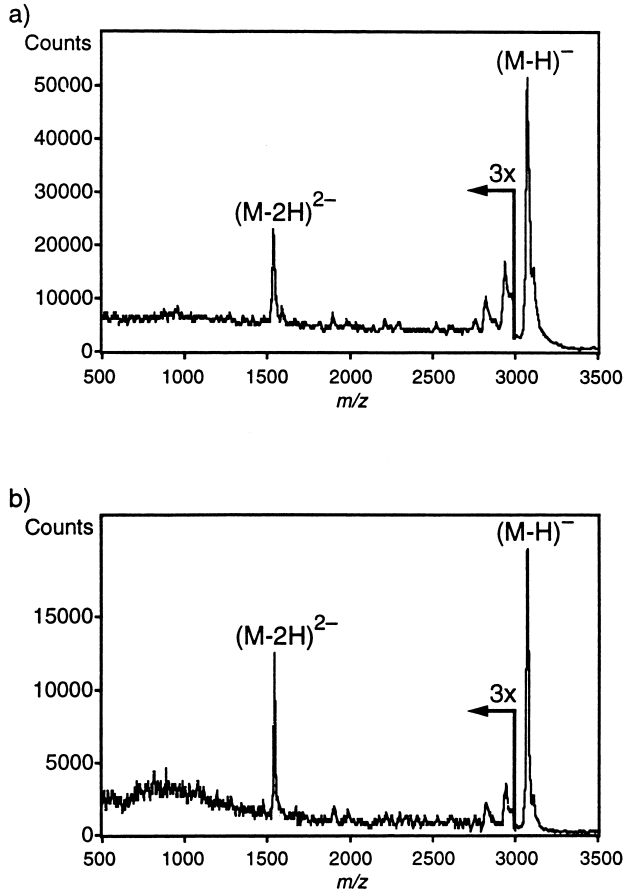

Figure 3. MALDI-MS mass spectra of d[(Ap)$_9$A] in the following co-matrix:matrix solutions: (**a**) 3 nmol:1.5 μmol piperidine:THAP and (**b**) 3 nmol:1.5 μmol triethylamine:THAP.

proton affinity of the co-matrix is less than the proton affinity of the nucleobase, the co-matrix is less effective at eliminating oligonucleotide fragmentation. This loss of efficiency can be overcome by increasing the mole fraction of co-matrix present in the solution. However, a co-matrix such as triethylamine with a high proton affinity is more effective at competing for the proton in the gas phase and can serve to nearly eliminate base-induced fragmentation in oligonucleotides.

To confirm that the reduction in oligonucleotide fragmentation seen in Figure 3 is due to the co-matrix interacting with the matrix and that this reduction is not due to an overall decrease in the molecular and fragment ion abundances, the same experiments were repeated using ATT as the matrix (Figure 4). Fragmentation is much more predominant in ATT than in either of the other two matrices investigated. Again, the utilization of a co-matrix of sufficiently high proton affinity can help to reduce fragmentation in oligonucleotides. Figure 4a is a mixture of 3 nmol of imidazole and 210 nmol of ATT. A significant number of backbone product ions are still observed in this mass spectrum. Increasing the amount of imidazole present in the solution to 300 nmol helps to reduce the number and abundance of these backbone fragments (Figure 4b). It

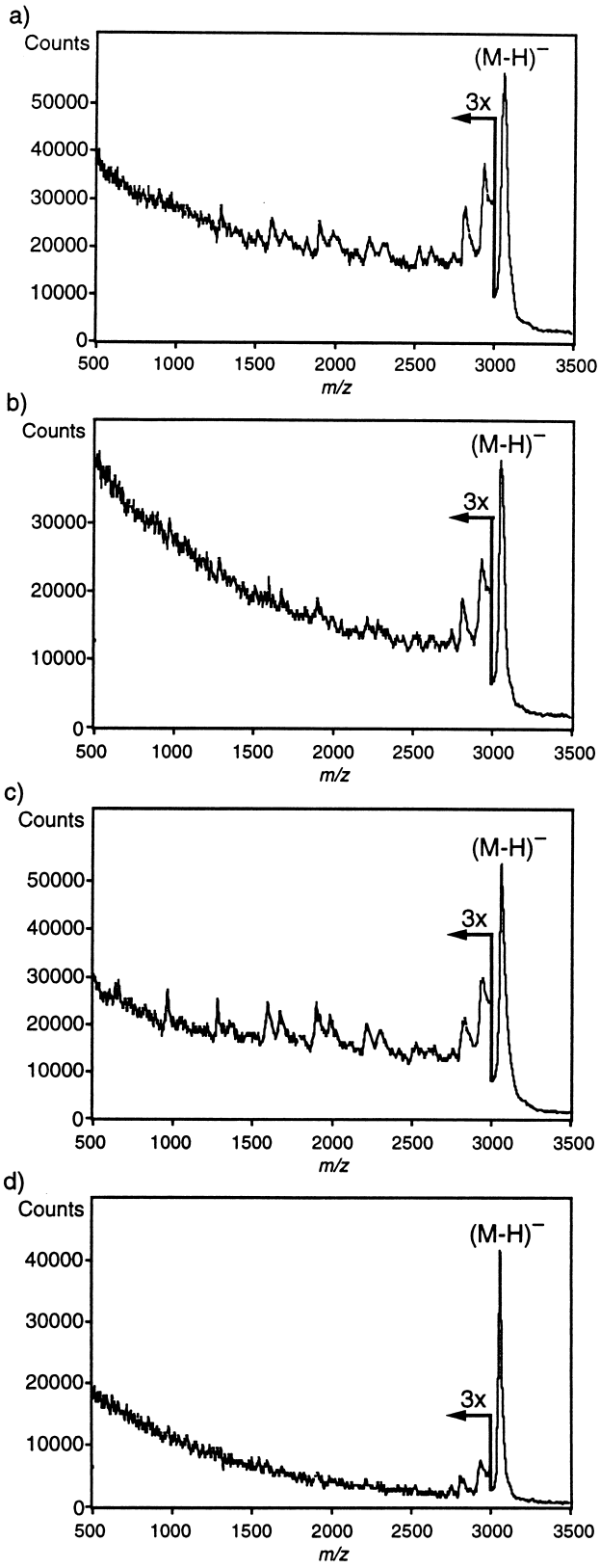

Figure 4. MALDI-MS mass spectra of d[(Ap)$_9$A] in the following co-matrix:matrix solutions: (**a**) 3 nmol:210 nmol imidazole:ATT, (**b**) 300 nmol:210 nmol imidazole:ATT, (**c**) 3 pmol:210 nmol triethylamine:ATT, and (**d**) 870 nmol:210 nmol triethylamine:ATT.

was found that further increasing the concentration of imidazole used did not result in any appreciable improvement in the mass spectral data and led to a reduction in the molecular ion abundance. The proton affinity of imidazole (225.2 kcal/mol) is lower than the proton affinity of deoxyadenosine (233.6 kcal/mol) and we attribute its inability to completely eliminate oligonucleotide fragmentation to this fact. In Figure 4c, 3 pmol of triethylamine was added to a 210 nmol solution of ATT. Again, a significant number of fragment ions are seen in this mass spectrum. However, as one would expect if the co-matrix proton affinity is the determining factor in reducing oligonucleotide fragmentation, increasing the mole ratio of triethylamine in the ATT matrix solution can nearly eliminate oligonucleotide fragmentation (Figure 4d).

These data demonstrate that improvements to the molecular ion stability can be rationalized in terms of the proton affinities of the co-matrices used. Triethylamine, which has the highest proton affinity, is the co-matrix most effective at reducing or eliminating metastable decomposition. Imidazole, which has the lowest proton affinity, is still capable of reducing metastable fragmentation presumably due to the fact that ATT is more acidic in the gas phase than the other matrices leading to increased fragmentation of the analyte. Even though imidazole has a proton affinity lower than that of deoxyadenosine, when present in high enough molar excess, it can serve as an additional sink for the acidic protons from the matrix thus reducing oligonucleotide fragmentation. We performed a number of additional investigations into the effect of co-matrix concentration and identity (data not shown), and all of the data is consistent with the supposition that the co-matrix serves as a proton sink upon laser desorption. As a proton sink, the co-matrix is protonated (preferentially) as compared to the oligonucleotide, thus improving the molecular ion stability of the analyte of interest. Furthermore, if the co-matrix is actively reducing gas-phase protonation of oligonucleotides, one would expect to see a reduction in molecular ion abundances and fragmentation in positive ion mode. Positive ion data of d[$(Ap)_9$A] with these co-matrices shows a reduction in both molecular ion abundance and fragmentation (data not shown), as one would expect for this mechanism.

Finally to confirm that the proton affinity of the co-matrix, rather than a gas-phase cooling process, is the determining factor in reducing oligonucleotide fragmentation during MALDI-MS, additional experiments were performed utilizing pyridine as a co-matrix. The proton affinity of pyridine is 222.3 kcal/mol, which is lower than the proton affinities of any of the nucleobases and the phosphodiester backbone. Based on our prior results, no reduction in oligonucleotide fragmentation would be expected using this co-matrix.

Typical results from the addition of pyridine as a co-matrix in an ATT matrix are shown in Figure 5. A large number of backbone fragment ions are detected

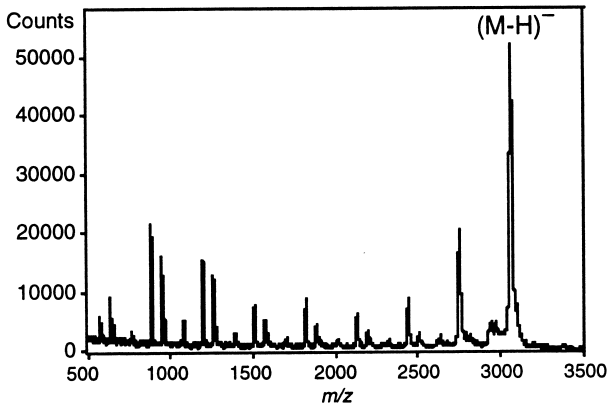

Figure 5. MALDI-MS mass spectra of d[$(Ap)_9$A] in a pyridine: ATT co-matrix:matrix solution.

with pyridine as the co-matrix, a result consistent with our proton sink mechanism rather than the gas-phase cooling mechanism. d[$(Tp)_9$T] did not exhibit any significant fragmentation when pyridine was used as a co-matrix as expected (data not shown). According to the mechanism proposed here, the proton affinity of pyridine is too low to effectively compete with the oligonucleotide for the protons from the matrix. Thus, base-protonation induced fragmentation of the oligonucleotide results.

The addition of a co-matrix of sufficiently high proton affinity can serve as a proton sink to reduce oligonucleotide fragmentation in MALDI-MS. As reported earlier [17], these co-matrices also reduce cation adducts present in the oligonucleotide sample, thereby providing a dual use of reducing cation adduction and oligonucleotide fragmentation. The addition of a co-matrix can adjust the matrix:analyte solution pH to values that yield increased molecular ion abundance as compared to spectra obtained without the use of a co-matrix, although the mole ratio of co-matrix added should be kept as low as possible to avoid decreases in the overall ion abundances. The information gained from these studies should facilitate further developments in matrix:co-matrix combinations for improved MALDI-MS analysis of oligonucleotides. We are also investigating the use of co-matrices as additives that improve fragment ion abundances for improved sequencing of oligonucleotides using MALDI-MS.

Acknowledgments

The authors thank Dr. Robert Hammer at LSU for the use of the oligonucleotide synthesizer. Financial support of this work was provided by the Louisiana Education Quality Support Fund [LEQSF(1996–99)-RD-A-08] and Louisiana State University.

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